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Selenoprotein W enhances skeletal muscle differentiation by inhibiting TAZ binding to 14-3-3 protein

Yeong Ha Jeon, Yong Hwan Park [1], Jea Hwang Lee, Jeong-Ho Hong, Ick Young Kim *

Division of Life Sciences, Korea University, 1, 5-Ka, Anam-Dong, Sungbuk-Ku, Seoul 136-701, Republic of Korea

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ABSTRACT

Selenoprotein W (SelW) is expressed in various tissues, particularly in skeletal muscle. We have previously reported that SelW is up-regulated during C2C12 skeletal muscle differentiation and inhibits binding of 14-3-3 to its target proteins. 14-3-3 reduces myogenic differentiation by inhibiting nuclear translocation of transcriptional co-activator with PDZ-binding motif (TAZ). Phosphorylation of TAZ at Ser89 is required for binding to 14-3-3, leading to cytoplasmic retention of TAZ and a delay in myogenic differentiation. Here, we show that myogenic differentiation was delayed in SelW-knockdown C2C12 cells. Down-regulation of SelW also increased TAZ binding to 14-3-3, which eventually resulted in decreasing translocation of TAZ to the nucleus. However, phosphorylation of TAZ at Ser89 was not affected. Although phosphorylation of TAZ at Ser89 was sustained by the phosphatase inhibitor okadaic acid, nuclear translocation of TAZ was increased by ectopic expression of SelW. This result was due to decreased binding of TAZ to 14-3-3. We also found that the interaction between TAZ and MyoD was increased by ectopic expression of SelW. Taken together, these findings strongly demonstrate that SelW enhances C2C12 cell differentiation by inhibiting TAZ binding to 14-3-3.

1. Introduction

Selenoprotein W (SelW) is the smallest selenoprotein (10 kDa) containing a selenocysteine (Sec, U) in a conserved – CXXU – motif, which corresponds to the – CXXC – redox motif of thioredoxin [1,2]. SelW may have anti-oxidant activity [3–5]. SelW is localized in the cytosol and is expressed in various tissues; however, it is particularly abundant in the skeletal muscle and brain of mammals [6–9]. SelW deficiency causes Keshan disease and white muscle disease in mammals [4,10,11]. We previously reported that SelW mRNA and protein expression is up-regulated during mouse skeletal muscle C2C12 cell differentiation by a MyoD-dependent increase in its promoter activity [12]. However, the precise function of SelW has not been determined during skeletal muscle C2C12 cell differentiation. We previously reported that SelW plays key roles in cell cycle progression by interacting with 14-3-3 [13]. SelW interacts with 14-3-3 to repress its interaction with target proteins including CDC25B and Rictor [13,14]. SelW has been identified as a 14-3-3 binding protein in a nuclear magnetic resonance (NMR) study [15,16].

14-3-3 is a family of regulatory molecules that plays important roles in functionally diverse signaling processes such as bacterial pathogenesis, neuronal development, and cell growth control through its interactions with various binding partners [17,18]. 14-3-3 is present in the brain, heart, liver, and testes [19,20]. It is clear that the function of many cellular proteins is modulated by binding to 14-3-3 [17]. 14-3-3 binds to phosphorylated protein ligands to regulate cell growth, cell cycle progression, and apoptosis [21–24]. 14-3-3 is also a regulator of cell differentiation by interacting with the transcriptional co-activator with PDZ-binding motif (TAZ) [25,26], and the transcriptional activity of TAZ is negatively regulated by its interaction with 14-3-3 [27–29]. Binding of TAZ to 14-3-3 reduces the transcriptional activity of TAZ for inducing target gene expression including myogenin, myosin heavy chain (MyHC) and muscle creatine kinase (MCK) [26].

TAZ was first identified as a 14-3-3 binding protein [30] involved in the muscle development [31]. Translocation of TAZ to the nucleus is required for its interaction with several transcription factors including MyoD, a key regulatory factor in skeletal muscle differentiation [26]. Transcriptional activity and nuclear translocation of TAZ is regulated by the Hippo signal pathway [28–30]. The Hippo pathway plays a key role in the control of organ size, cell proliferation, and differentiation. The signaling components large tumor suppressor (LATS) and protein phosphatase 1 (PP1) regulate nuclear translocation, transcriptional activity, and stability of TAZ [28,29]. Transcriptional activity and nuclear translocation of TAZ is inhibited by LATS. LATS phosphorylates TAZ on several serine residues including Ser89 [27]. Phosphorylation of TAZ at Ser89 by LATS leads to its cytoplasmic retention and transcriptional

Abbreviations: TAZ, transcriptional co-activator with PDZ-binding motif; NMR, nuclear magnetic resonance spectroscopy; MyHC, myosin heavy chain; MCK, muscle creatine kinase; LATS, large tumor suppressor; PP1, protein phosphatase 1; Sec, U, selenocysteine

* Corresponding author. Tel./fax: +82 2 3290 3449.
E-mail address: ickkim@korea.ac.kr (I.Y. Kim).
[1] Present address: Inflammatory Disease Section, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA.

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inactivation by enhancing the interaction with 14-3-3 [29]. A mutant TAZ, in which the Ser is replaced with Ala (S89A mutant), disrupts the interaction with 14-3-3, leading to increased translocation of TAZ to the nucleus [25,27,30]. PP1 is a serine/threonine phosphatase involved in various cellular processes [32]. PP1 de-phosphorylates TAZ at Ser89. De-phosphorylation of TAZ at Ser89 by PP1 promotes translocation of TAZ to the nucleus, leading to its transcriptional activity [29,33]. Additionally, ectopic expression of PP1 attenuates the interaction between TAZ and 14-3-3 and increases translocation of TAZ to the nucleus through de-phosphorylation of TAZ at Ser89 [33]. However, the molecular mechanism regulating TAZ remains unknown. Because TAZ nuclear translocation and transcriptional activity are negatively regulated by the interaction with 14-3-3, which also interacts with SelW, we sought to determine the function of the complex between SelW and 14-3-3 for regulating TAZ nuclear translocation during skeletal muscle C2C12 cell differentiation. In this study, we have shown that the complex formation between SelW and 14-3-3 was increased during C2C12 cell differentiation compared to that during cell proliferation, whereas binding of TAZ to 14-3-3 was decreased during C2C12 cell differentiation. We also found that down-regulation of SelW reduced C2C12 cell differentiation due to increased interaction between TAZ and 14-3-3, which decreased translocation of TAZ to the nucleus, whereas nuclear translocation of TAZ was increased by ectopic expression of SelW, which resulted from inhibiting TAZ binding to 14-3-3. However, TAZ phosphorylation at Ser89 was not affected by either over-expression or down-regulation of SelW. Although phosphorylation of TAZ at Ser89 was retained by okadaic acid (OA), binding of TAZ to 14-3-3 was inhibited by ectopic expression of SelW. Additionally, ectopic expression of SelW increased binding of TAZ to MyoD. Taken together, these results suggested that SelW elevated skeletal muscle C2C12 cell differentiation by interrupting the interaction between TAZ and 14-3-3.

2. Materials and methods

2.1. Cell culture and transfection

Mouse skeletal muscle C2C12 (CRL-1722) myoblast cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were propagated in growth medium (GM) consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) at 37 °C in 5% CO_2. To induce differentiation, the cells were cultured to ~100% confluence in GM followed by an exchange to differentiation medium (DM) consisting of DMEM containing 2% heat-inactivated horse serum. The DM was exchanged every day. Human embryonic kidney 293 (HEK293) cells were grown in DMEM containing 10% FBS at 37 °C in 5% CO_2. The cells were seeded at a density of 3×10^5 cells in 60-mm dishes for transient transfection. Twelve hours after seeding, the cells were transfected using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions [12].

2.2. RNA interference and plasmids

The siSelW used in this study was designed by Invitrogen. The sequence for mouse siSelW was as follows: siSelW 5′-CCG GAA ACU GGU GAC CGC CAU CAA A-3′. A point mutation in SelW at Ser13 to Cys was generated by site-directed mutagenesis of the cDNA by polymerase chain reaction (PCR) using the pcDNA 3.1 +/SelW plasmid [12,13]. The HA-14-3-3β and Flag-TAZ plasmids were kindly provided [25].

2.3. Antibodies and western blotting

The cells were washed twice with PBS and lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 3 mM dithiothreitol (DTT), phosphatase inhibitor 1 mM NaF, and 1 mM Na3VO4. The proteins from whole-cell lysates were separated by 6–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to a membrane that was blocked with 5% skim milk for 1 h and probed with specific antibodies. The antibodies were obtained from the following sources: anti-TAZ and anti-phospho-TAZ (Ser89) were kindly provided by Prof. J. H. Hong (Korea University); anti-MyoD was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or from Pharmingen (BD Bioscience, San Jose, CA, USA); anti-myogenin was from Pharmingen (BD Bioscience); anti-MyHC and anti-Flag were from Sigma (Sigma-Aldrich, St. Louis, MO, USA); anti-His and anti-HA were from ABM (Richmond, BC, Canada); and anti-α-tubulin was obtained from AB Frontier (Daejeon, Republic of Korea). The cell lysates were incubated with LDS sample buffer and reducing reagent (Invitrogen) at 70 °C for 10 min to detect SelW. The lysates were then separated on a 12% Bis-Tris gel (Invitrogen), transferred to a membrane, and probed with the SelW antibody. HRP-conjugated secondary antibodies were incubated with the membranes for 1 h [13].

2.4. Quantitative PCR

Total RNA was isolated from C2C12 cells using TRIzol reagent (Invitrogen). The cDNA was amplified with LightCycler 480 SYBR Green I Master (Roche Diagnostics, Manheim, Germany). The primers for the gene sequences were designed between different exons to prevent DNA amplification. The following forward and reverse primers were used: for SelW, 5′-GTG TAT TGT GGA GCT TGA GGC-3′ and 5′-CCA AGG CAG CTT TGA TGG CGG-3′; GAPDH, 5′-AGG TCG GAG TCA ACG GAT TT-3′ and 5′-AGG TGG AGG AGT GGG TGT CG-3′.

2.5. Immunoprecipitation

Cells were lysed with immunoprecipitation buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 20 mM NaF, and 2 mM Na3VO4. The lysates were mixed with antibodies overnight, and the immune complexes were then incubated with Protein A or G beads for 1.5 h. The beads were washed and boiled 2× with SDS sample buffer for 3 min. The samples were loaded onto SDS-PAGE gels, transferred to a membrane, and incubated with primary antibody at 4 °C for overnight. After a further incubation with HRP-conjugated secondary antibody for 1 h at room temperature, immunoreactive bands were visualized using a West Pico Enhanced ECL Detection kit (Pierce, Rockford, IL, USA) [34].

2.6. Nuclear fraction

C2C12 cells were washed with cold PBS twice and were harvested by scrapping and centrifugation at 850 ×*g* for 5 min at 4 °C. After the supernatant was removed, the cells were resuspended in lysis buffer containing 10 mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After gentle vortexing, the cells were incubated on ice for 15 min, and then 10% NP-40 was added to the samples at a final concentration of 0.5%. After thoroughly vortexing the samples for 10 sec, the lysates were centrifuged at 16,000 ×*g* for 2 min at 4 °C. The supernatants were collected as the cytosolic extract, and the pellets were washed twice with lysis buffer. The pellets were resuspended in nuclear extraction buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin and vortexed strongly for 10 min at 4 °C. After centrifugation at 16,000 ×*g* for 30 min at 4 °C, the soup was collected as the nuclear extract [13].

2.7. Confocal microscopy

Cells on coverslips were washed with cold PBS, fixed with 3.7% formaldehyde for 15 min at room temperature (RT), permeabilized with 0.1% Triton X-100 for 5 min, and incubated with 2% BSA overnight to block nonspecific staining. The cells were then immunostained with antibodies in 0.1% BSA for 1 h at RT, washed three times with PBS, and further incubated with fluorescence conjugated secondary antibodies (FITC) for 1.5 h at RT. The cells were stained with DAPI for 3 min to visualize nuclei. Finally, the cells were mounted on slides using mounting solution. Immunofluorescence was examined using a fluorescence microscope (Zeiss LSM 510 META; Oberkochen, Germany).

3. Results

3.1. SelW is involved in C2C12 cell differentiation

The myogenic differentiation program can be triggered in C2C12 myoblasts by shifting confluent C2C12 cells from GM to DM. To induce differentiation, C2C12 myoblasts were maintained in GM up to ~100% confluence (D0). After exchanging the medium with DM, differentiating C2C12 cells were observed at 1, 3, and 5 days of differentiation (D1, D3 and D5, respectively) [12]. Protein expression of well-known muscle-specific markers such as MyoD, myogenin, and MyHC was confirmed during C2C12 cell differentiation. Expression of MyoD, myogenin, and

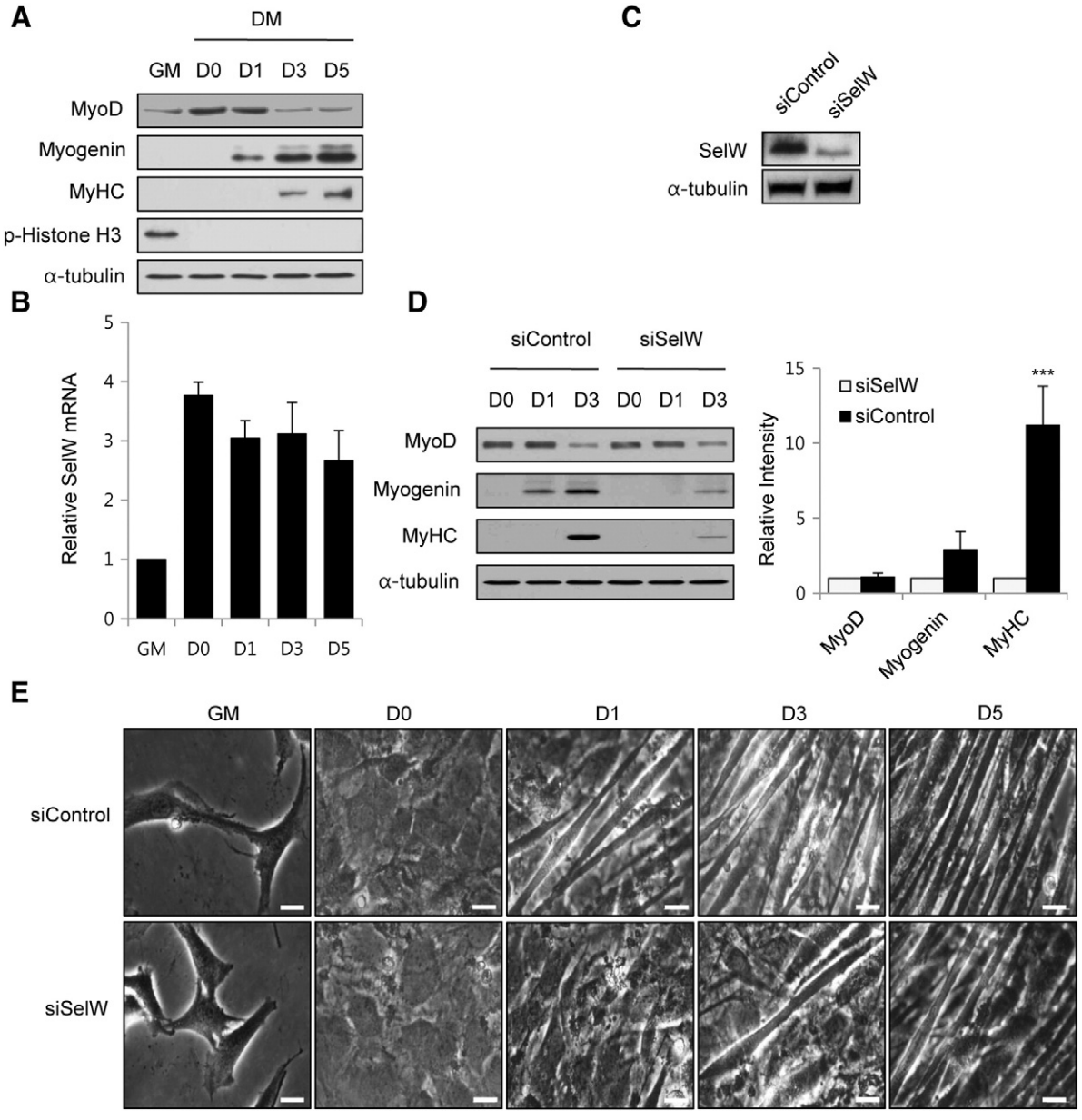

Fig. 1. SelW is up-regulated during C2C12 cell differentiation. (A) Expression of muscle-specific marker proteins during C2C12 cell differentiation. C2C12 cell differentiation was induced. The cells were harvested at the indicated time points, and were then analyzed by western blot using the indicated antibodies. α-Tubulin served as the loading control. (B) Quantitative PCR was carried out with SelW specific primers. Error bars represent ± standard deviation (s.d.). (C) C2C12 cells were transiently transfected with siControl or siSelW. The cells were harvested at D1 after transfection with siSelW. SelW protein was analyzed by western blot. α-Tubulin served as the loading control. (D) C2C12 cells were transiently transfected with siControl or siSelW. The cells were harvested at the indicated time points after transfection and were analyzed by western blot using the indicated antibodies. α-Tubulin served as the loading control (left). The quantity of MyoD, myogenin and MyHC expression represents the average values at D3. The graph indicates the results from three independent experiments (***$p < 0.001$). Error bars represent ± standard deviation (s.d.), and the p value compares the siControl to siSelW (right). (E) SelW-knockdown C2C12 cells were established and used for myogenic differentiation. Microscopic observations were conducted at the indicated time points. Scale bar: 20 μm.

MyHC was increased during C2C12 cell differentiation. Phospho-histone H3, a cell proliferation marker, was only detected in C2C12 myoblasts cultured in GM (Fig. 1A). As shown in a previous report [12], we reconfirmed that the expression of SelW mRNA was up-regulated during C2C12 cell differentiation, which was determined by quantitative PCR (Fig. 1B). Because SelW mRNA expression was increased during C2C12 cell differentiation, we next investigated whether down-regulation of SelW might inhibit C2C12 cell differentiation. Thus, we examined MyoD, myogenin, and MyHC expression levels during myogenic differentiation in SelW-knockdown C2C12 cells. We preferentially observed that SelW expression was efficiently reduced by SelW-specific siRNA (Fig. 1C). The expression levels of the muscle-specific gene markers myogenin and MyHC was decreased during C2C12 cell differentiation of SelW-knockdown cells compared to that in control cells, whereas MyoD expression remained unchanged (Fig. 1D). We also found that the level of myotube formation was reduced in SelW-knockdown C2C12 cells, compared to control cells (Fig. 1E). These results indicate that SelW is involved in C2C12 cell differentiation.

3.2. TAZ binding to 14-3-3 is diminished during myogenic differentiation

TAZ translocation to the nucleus is required to elevate the expression of genes such as myogenin, MyHC, and MCK and stimulate myogenic differentiation [26]. We found that translocation of TAZ to the nucleus was increased during C2C12 cell differentiation (Fig. 2A). Cytoplasmic retention and transcriptional inactivation of TAZ is caused by its interaction with 14-3-3 [30]. To investigate the interaction between TAZ and 14-3-3 during C2C12 cell differentiation, we overexpressed Flag-tagged TAZ and HA-tagged 14-3-3 for an in vitro interaction study. We observed binding of TAZ to 14-3-3 (Fig. 2B), which decreased during C2C12 cell differentiation (Fig. 2C). An immunofluorescence confocal microscopic analysis revealed that TAZ was translocated to the nucleus under differentiating conditions (Fig. 2D). These results indicate that inhibiting the interaction between TAZ and 14-3-3 is required for nuclear translocation of TAZ.

3.3. Binding of SelW to 14-3-3 affects nuclear translocation of TAZ

We next investigated whether SelW was involved in translocation of TAZ to the nucleus for myogenic differentiation. We found that down-regulation of SelW resulted in a decrease in TAZ translocation to the nucleus during C2C12 differentiation compared to that of control cells (Fig. 3A). To understand the interaction studies of SelW in skeletal muscle differentiation, we expressed a mutant SelW where Sec was changed to Cys (U13C). Expression of recombinant wild type SelW was very difficult to work with, as the codon for Sec, UGA, was often recognized as a stop codon. Thus, we used a mutant SelW, in which the Sec was replaced with a Cys (U13C). Although SelW modulated translocation of TAZ to the nucleus, it did not interact with TAZ (Fig. 3B). To investigate how nuclear translocation of TAZ was decreased by down-regulating SelW, we analyzed the TAZ translocation regulatory pathway from the cytoplasm to the nucleus. It has been reported that translocation of TAZ to the nucleus is regulated by complex formation between TAZ and 14-3-3 [30]. We have previously reported that SelW interrupts the interaction between 14-3-3 and its target proteins [13,14]. Therefore, we investigated whether SelW regulated translocation of TAZ from the cytoplasm to the nucleus by interrupting its interaction with 14-3-3. We found that binding of TAZ to 14-3-3 was significantly increased in SelW-knockdown C2C12 cells compared to that in control cells but did not affect phosphorylation of TAZ at Ser89 (Fig. 3C). As expected, binding of SelW to 14-3-3 was increased during C2C12 cell differentiation (Fig. 3D). We then investigated subcellular location of TAZ and 14-3-3. Confocal microscopic observations revealed that almost all of the TAZ was translocated to the nucleus compared to that in SelW-knockdown cells, which was confirmed by immunofluorescence staining (Fig. 3E

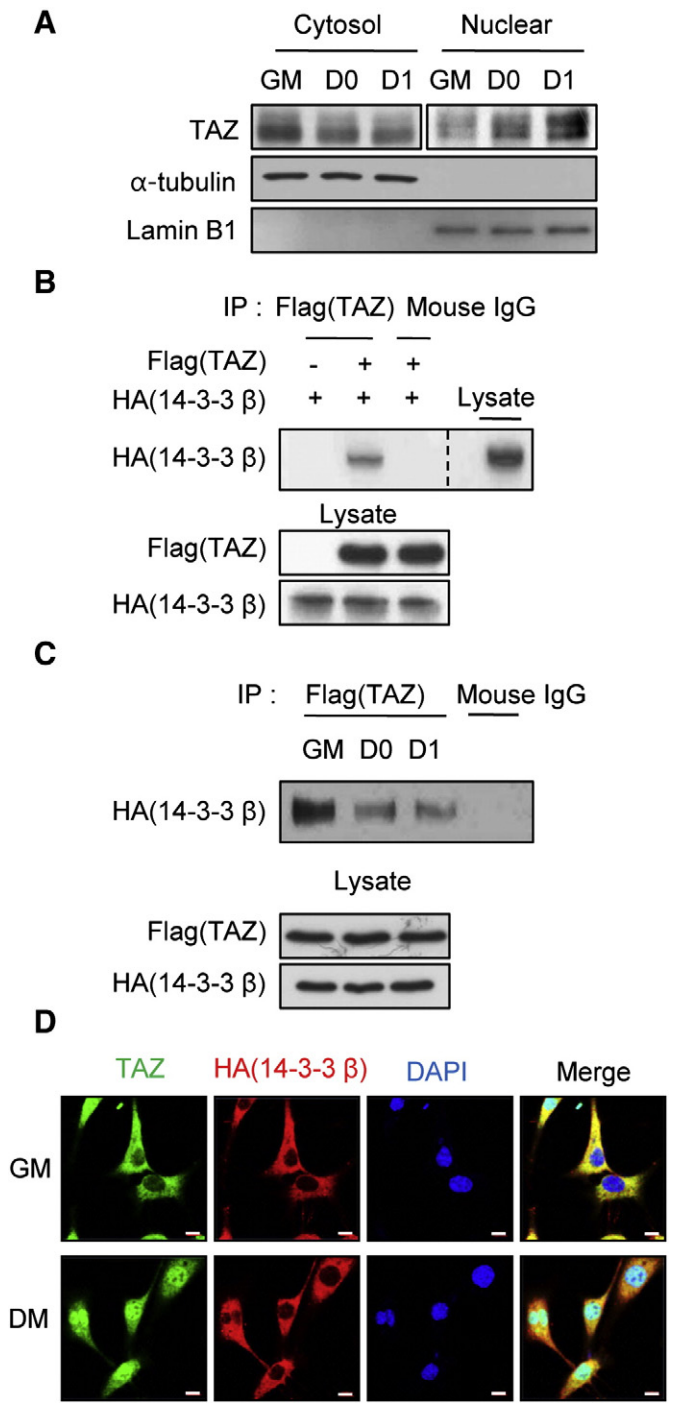

Fig. 2. Inhibition of 14-3-3 binding to TAZ is required for TAZ nuclear translocation. (A) Nuclear translocation of TAZ during C2C12 cell differentiation. The cytoplasmic and nuclear fractions were obtained at the indicated time points. Samples were probed for α-tubulin as a cytoplasmic marker and lamin B1 as a nuclear marker. (B) C2C12 cells were co-transfected with Flag-TAZ or HA-14-3-3β, and the cells were harvested 24 h after transfection. Cell extracts were immunoprecipitated with anti-Flag or anti-Mouse IgG antibodies and were immunoblotted with anti-HA antibody. (C) C2C12 cells were co-transfected with Flag-TAZ or HA-14-3-3β and the cells were harvested at the indicated time points. Cell extracts were immunoprecipitated with anti-Flag and were immunoblotted with anti-HA antibody. (D) C2C12 cells were transfected with HA-14-3-3β. After transfection, the cells were cultured in GM or DM. The cells were stained with anti-TAZ antibody and anti-HA antibody and then incubated with secondary IgG linked with Alexa Fluor 488 (green, TAZ) or Alexa Fluor 594 (red, HA-14-3-3β). DNA was stained with DAPI (blue). Images of the samples were obtained using a confocal microscope. Scale bar: 10 μm.

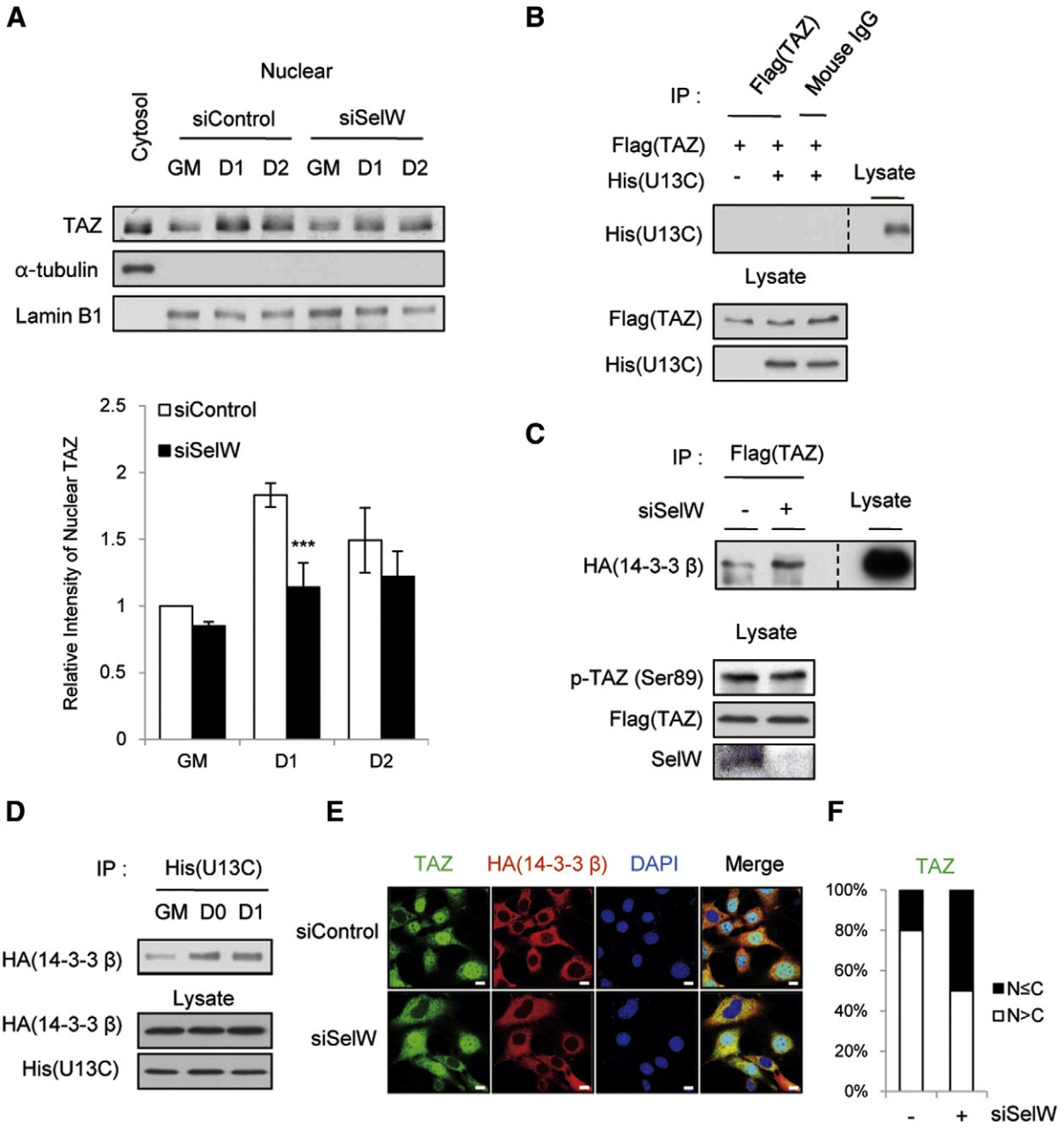

Fig. 3. Binding of SelW to 14-3-3 is required for nuclear translocation of TAZ. (A) C2C12 cells were transiently transfected with siControl or siSelW. Cytoplasmic and nuclear fractions were obtained at the indicated time points after transfection and immunoblotted using the TAZ antibody. Samples were probed for α-tubulin as a cytoplasmic marker and lamin B1 as a nuclear marker (top). The quantity of nuclear TAZ represents the average values at the indicated time points. The graph indicates the results from three independent experiments (***$p < 0.001$). Error bars represent ± standard deviation (s.d.), and the p value compares the siControl to siSelW (bottom). (B) C2C12 cells were co-transfected with Flag-TAZ or His-SelW(U13C), and the cells were harvested 24 h after transfection. The cell extracts were immunoprecipitated with anti-Flag and were then immunoblotted with anti-His antibody. (C) C2C12 cells were co-transfected with siSelW, Flag-TAZ, or HA-14-3-3β. The cells were harvested 24 h after transfection, immunoprecipitated with anti-Flag antibody, and then immunoblotted using the indicated antibodies. (D) C2C12 cells were co-transfected with His-SelW(U13C) and HA-14-3-3β. The cells were harvested at the indicated time points, immunoprecipitated with anti-His or anti-Mouse IgG antibodies, and were then immunoblotted using the indicated antibodies. (E) C2C12 cells were co-transfected with siControl or siSelW and HA-14-3-3β. The cells were cultured for D1. The cells were stained with anti-TAZ and anti-HA antibodies and then incubated with secondary IgG linked with Alexa Fluor 488 (green, TAZ) or Alexa Fluor 594 (red, HA-14-3-3β). DNA was stained with DAPI (blue). Images of the samples were obtained using a confocal microscope. Scale bar: 10 μm. (F) Nuclear translocation of TAZ proteins in (E) was quantitatively analyzed according to whether it was higher in the nucleus (N > C), higher in the cytoplasm (N < C) or evenly distributed between the nucleus and cytoplasm (N = C).

and F). These observations indicate that SelW plays a key role in translocation of TAZ to the nucleus.

3.4. SelW promotes nuclear translocation of TAZ

We investigated whether ectopic expression of SelW(U13C) inhibited binding of TAZ to 14-3-3 and promoted nuclear translocation of TAZ to stimulate myogenic differentiation. Thus, we examined TAZ binding to 14-3-3 with or without SelW(U13C) co-expression. We observed that ectopic expression of SelW(U13C) decreased binding of TAZ to 14-3-3 compared to that in control cells but did not affect phosphorylation of TAZ at Ser89 (Fig. 4A). Furthermore, translocation of TAZ to the nucleus was increased by ectopically expressing SelW(U13C) (Fig. 4B). When the cells were treated with OA, which is an inhibitor of PP1 and PP2A [33,35], phosphorylation of TAZ at Ser89 was significantly increased with increasing amounts of OA (Fig. 4C), and TAZ binding to 14-3-3 was increased by treatment with OA. Despite OA treatment, ectopic expression of SelW(U13C) significantly inhibited binding of TAZ to

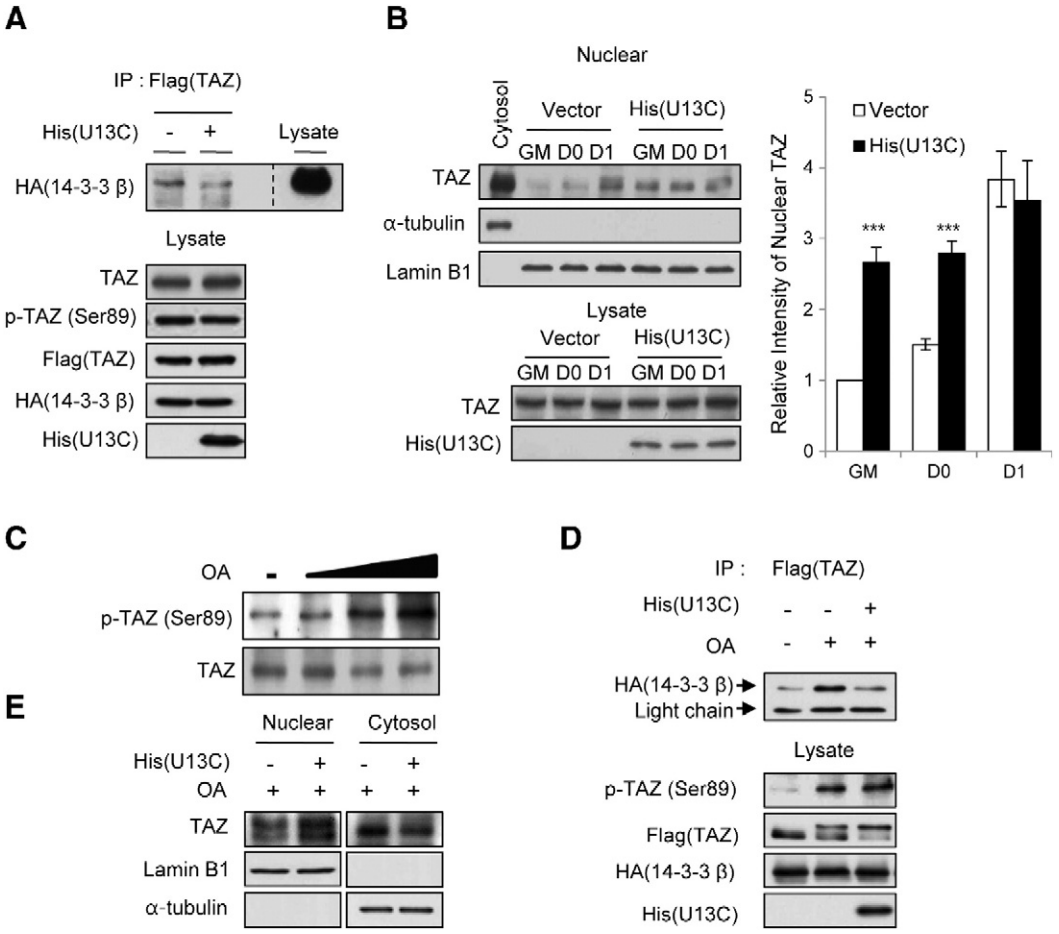

Fig. 4. Nuclear translocation of TAZ is promoted by SelW. (A) C2C12 cells were co-transfected with Flag-TAZ, HA-14-3-3β or His-SelW(U13C) The cells were harvested 24 h after transfection, immunoprecipitated with anti-Flag antibody, and immunoblotted using the indicated antibodies. (B) C2C12 cells were transfected with empty vector or His-SelW(U13C). Cytoplasmic and nuclear fractions were obtained at the indicated time points after transfection and immunoblotted using the TAZ antibody. Samples were probed for α-tubulin as a cytoplasmic marker and lamin B1 as a nuclear marker, and analyzed by western blot using the indicated antibodies (left). The quantity of nuclear TAZ represents the average values at the indicated time points. The graph indicates the results from three independent experiments (***$p < 0.001$). Error bars represent ± standard deviation (s.d.), and the p value compares the vector to His(U13C) (right). (C) C2C12 cells were treated without or with 10, 50 and 100 ng/ml of okadaic acid (OA). After a 6 h treatment, the cells were harvested and analyzed by western blot using the indicated antibodies. (D) C2C12 cells were co-transfected with His-SelW(U13C), Flag-TAZ, and HA-14-3-3β. At 24 h after transfection, the cells were treated with 50 ng/ml of OA for 6 h. The cells were immunoprecipitated with anti-Flag antibody, and immunoblotted using the indicated antibodies. (E) C2C12 cells were transfected with empty vector or His-SelW(U13C), and were incubated with 25 ng/ml of OA for 12 h prior to harvest. The cytoplasmic and nuclear fractions were obtained at D1 and immunoblotted using the TAZ antibody. Samples were probed for α-tubulin as a cytoplasmic marker and lamin B1 as a nuclear marker.

14-3-3 and increased nuclear translocation of TAZ (Fig. 4D and E). Collectively, these data suggest that SelW inhibits binding of TAZ to 14-3-3 and promotes translocation of TAZ to the nucleus.

3.5. SelW enhances myogenic differentiation by inhibiting TAZ binding to 14-3-3

We next investigated whether ectopic expression of SelW(U13C) promoted myogenic differentiation. As shown in Fig. 5A, ectopic expression of SelW(U13C) elevated the expression of myogenin but did not affect the expression of MyoD (Fig. 5A). Furthermore, when TAZ phosphorylation was sustained by OA treatment, myogenin expression was increased by SelW (Fig. 5B). Next, we examined whether ectopic expression of SelW in endogenous SelW-knockdown C2C12 cells could enhance myogenic differentiation. We knocked down SelW in C2C12 cells and then ectopically expressed SelW(U13C). Ectopic expression of SelW(U13C) restored the expression of myogenin that was decreased by SelW-specific siRNA (Fig. 5C). Binding of TAZ to 14-3-3 was decreased by ectopic expression of SelW(U13C) (Fig. 5D). We also found that binding of TAZ to 14-3-3 was decreased with increasing amounts of SelW(U13C) (Fig. 5E). These results clearly demonstrate that SelW enhances myogenic differentiation by inhibiting TAZ binding to 14-3-3.

3.6. Binding of TAZ to MyoD is increased by the action of SelW

Most TAZ is translocated to the nucleus during skeletal muscle cell differentiation to physically interact with MyoD. This promotes MyoD-induced myogenin and MCK expression for skeletal muscle cell differentiation [26]. To investigate TAZ binding to MyoD, we overexpressed Flag-tagged TAZ and HA-tagged MyoD and observed binding of TAZ to MyoD (Fig. 6A). Translocation of TAZ to the nucleus was reduced during SelW-knockdown C2C12 cell differentiation (Fig. 3A), whereas ectopic expression of SelW(U13C) promoted TAZ translocation to the nucleus (Fig. 4B). Therefore, we next investigated whether SelW(U13C) regulated the interaction between TAZ and MyoD. We examined binding of TAZ to MyoD during ectopic expression of SelW(U13C). The interaction between TAZ and MyoD was increased by ectopic expression of SelW(U13C) (Fig. 6B). These results suggest that SelW is essential for skeletal muscle differentiation by increasing TAZ binding to MyoD. Fig. 7 is a model of myogenic differentiation regulated by SelW.

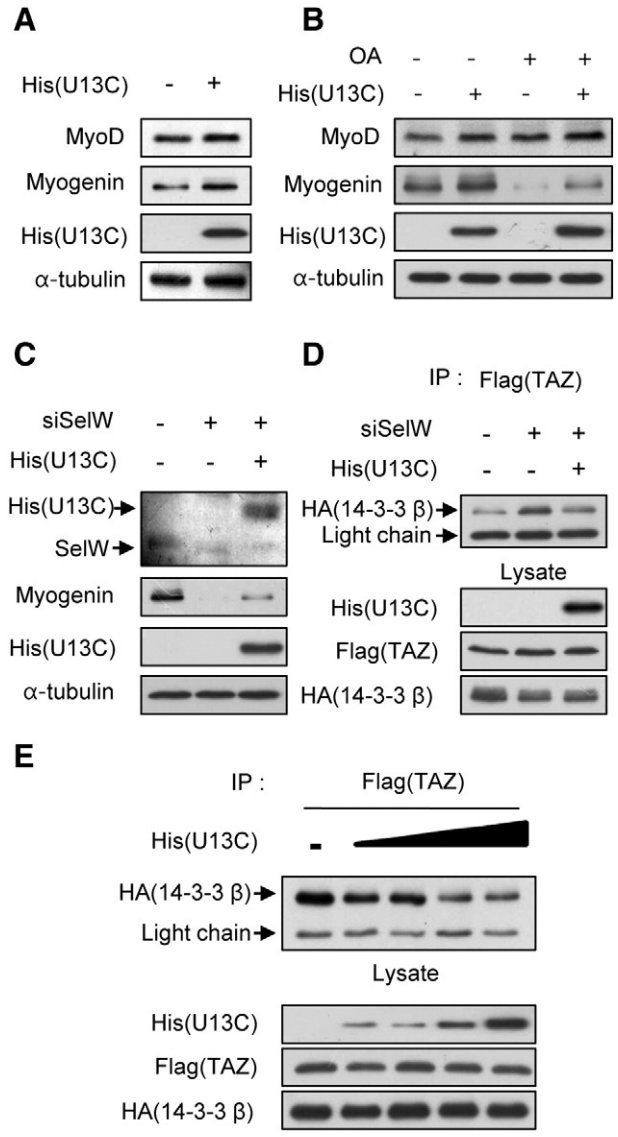

Fig. 5. Enforced expression of SelW enhances C2C12 differentiation. (A) C2C12 cells were transfected with empty vector or His-SelW(U13C). The cells were harvested at D1 after transfection and were analyzed by western blot using the indicated antibodies. (B) C2C12 cells were transfected with empty vector or His-SelW(U13C) and treated with 25 ng/ml of OA for 12 h prior to harvest. The cells were harvested at D1 and analyzed by western blot using the indicated antibodies. (C) Electroporation of C2C12 cells was performed using a Neon transfection system according to the manufacturer's instructions for transient transfection with siSelW. Twelve hours after transfection with siSelW, the cells were co-transfected with His-SelW(U13C) using Lipofectamine 2000 transfection reagent. The cells were harvested at D1 and analyzed by western blot using the indicated antibodies. (D) Electroporation of C2C12 cells was performed using a Neon transfection system according to the manufacturer's instructions for transient transfection with siSelW. Twelve hours after transfection with siSelW, the cells were co-transfected with His-SelW(U13C), Flag-TAZ and HA-14-3-3β using Lipofectamine 2000 transfection reagent. Twenty-four hours after transfection, the cells were immunoprecipitated with anti-Flag antibody, and immunoblotted using the indicated antibodies. (E) HEK293 cells were co-transfected with Flag-TAZ, HA-14-3-3β and increasing amount of 0, 0.25, 0.5, 1, and 2 μg g His-SelW(U13C). Twenty-four hours after transfection, the cells were immunoprecipitated with anti-Flag antibody, and immunoblotted using the indicated antibodies.

4. Discussion

SelW is involved in growth and proliferation of C2C12 skeletal muscle [9]. In this study, we investigated the potential role of SelW to regulate C2C12 skeletal muscle differentiation. We found that SelW mRNA expression was up-regulated during C2C12 cell differentiation (Fig. 1B), whereas down-regulation of SelW significantly diminished C2C12 cell differentiation, which resulted from a reduction in the

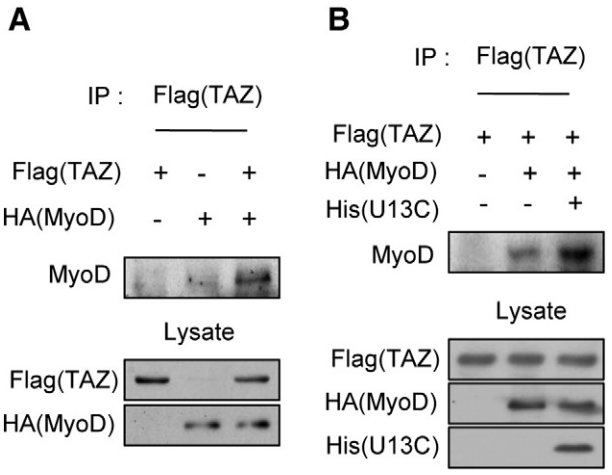

Fig. 6. SelW enhances TAZ binding of to MyoD. (A) HEK293 cells were co-transfected with Flag-TAZ or HA-MyoD, and the cells were harvested 24 h after transfection. Cell extracts were immunoprecipitated with anti-Flag antibody and immunoblotted using the indicated antibodies. (B) C2C12 cells were co-transfected with Flag-TAZ, HA-MyoD, or His-SelW(U13C) and harvested at D1 after transfection. Cell extracts were immunoprecipitated with anti-Flag antibody and immunoblotted using the indicated antibodies.

expression of myogenin and MyHC but not MyoD (Fig. 1D and E). We have previously reported that SelW mRNA and protein expression levels are elevated by the interaction between a specific E-box of SelW and MyoD during early skeletal muscle differentiation [12]. MyoD is essential for skeletal muscle differentiation [36] and plays a key role in the expression of muscle-specific genes [37]. Binding of MyoD to target proteins including TAZ is required for expression of the muscle-specific genes myogenin and MCK [26].

Nuclear translocation of TAZ is required for the interaction with MyoD [26]. Nuclear translocation of TAZ is regulated by LATS, which phosphorylates TAZ at Ser66, 89, 117, and 311 [27]. In particular, TAZ

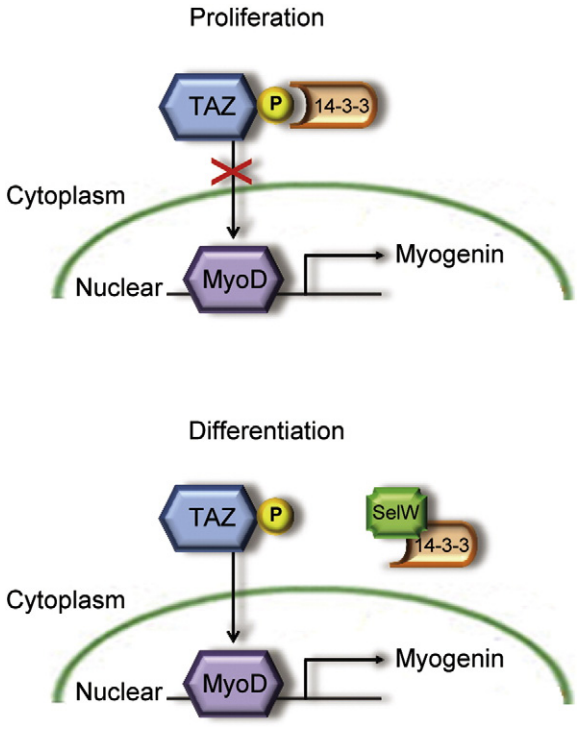

Fig. 7. A regulatory mechanism of SelW in skeletal muscle differentiation by inhibiting TAZ binding to 14-3-3.

transcriptional activity has been associated with phosphorylation at Ser89 and Ser311 [29]. Phosphorylation at Ser311 mediates polyubiquitination and degradation of TAZ through interaction with $SCF^{\beta\text{-}TrCP}$ E3 ligase [28,29,38]. The Ser89 in TAZ generates a 14-3-3 binding site [28]. TAZ binding to 14-3-3 is inhibited with increasing amounts of the serine/threonine inhibitor K252a. A 14-3-3 mutant that disrupts the E180K phosphopeptide-binding pocket and K49E eliminates TAZ binding. Thus, phosphorylation of TAZ by a serine/threonine kinase is required for its interaction with 14-3-3 [17,30,39–41]. AKT is another serine/threonine kinase involved in various cellular processes [42,43]. AKT is known to phosphorylate YAP which shares 50% sequence with TAZ [27,44]. We have reported that SelW enhances activity of the mTORC2/AKT pathway for AKT phosphorylation at Ser473 by inhibiting 14-3-3 binding to Rictor [14]. This raises the question of whether TAZ phosphorylation is increased due to AKT activity during C2C12 muscle differentiation. However, phosphorylation of TAZ at Ser89 is regulated by LATS not AKT [45]. Replacing Ser89 with Ala (S89A) in TAZ completely abolishes the interaction with 14-3-3, and nuclear translocation of the mutant TAZ is more than that of the wild-type protein [25,27,30].

The increased binding of TAZ at Ser89 to 14-3-3 reduces nuclear translocation and transcriptional activity of TAZ [28]. Binding of TAZ to 14-3-3 decreased during C2C12 cell differentiation, which resulted in translocation of TAZ to the nucleus (Fig. 2). Nuclear translocation of TAZ is required for skeletal muscle differentiation and interaction with MyoD. The transcription activity of the MyoD-mediated genes myogenin and MCK is promoted by the interaction with TAZ, and myogenin expression is increased by inducing the expression of TAZ in regenerating muscle after injury. The interaction between MyoD and a promoter of myogenin was attenuated upon down-regulation of TAZ, which resulted in diminished myogenic differentiation. In addition, the WW domain of TAZ (aa 1–163) is required for interaction with the C-terminal (aa 162–318) of MyoD [26].

We have reported that SelW regulates binding of 14-3-3 to its target proteins, CDC25B and Rictor, to enhance cell growth [13,14]. Binding of 14-3-3 to CDC25B and Rictor is increased significantly in SelW-knockdown cells, and ectopic expression of SelW reduces these interactions [13,14]. As shown in Fig. 3A, SelW-knockdown cells reduced translocation of TAZ to the nucleus compared to that of control cells (Fig. 3A) because down-regulation of SelW increased TAZ binding to 14-3-3 (Fig. 3C). The observation that down-regulation of SelW increased TAZ binding to 14-3-3 is very intriguing. Although SelW did not interact with TAZ (Fig. 3B), it is possible that SelW may be a TAZ regulatory molecule during C2C12 cell differentiation.

PP1 is a serine/threonine phosphatase involved in various cellular processes [46]. Phosphorylation of TAZ at Ser89 is decreased by PP1. The ectopic expression of PP1 significantly reduced the binding of TAZ to 14-3-3, which resulted in an increase in the translocation of TAZ to the nucleus. PP1 expression is reduced by OA with an IC_{50} of 20 nM [33,35]. Phosphorylation of TAZ at Ser89 and its interaction with 14-3-3 was increased significantly following treatment with OA (Fig. 4C and D). Ectopic expression of SelW decreased the binding of TAZ to 14-3-3 that was increased by treatment with OA (Fig. 4A and D). Furthermore, translocation of TAZ to the nucleus was increased by SelW (Fig. 4B and E). Therefore, our findings suggest that SelW inhibits TAZ binding to 14-3-3 and promotes nuclear localization of TAZ.

In addition to our recent findings [13,14], increasing amounts of SelW decreased the binding of 14-3-3 to its target protein TAZ (Fig. 5C). 14-3-3 plays widespread roles in various signaling processes such as cell growth and development through its interaction with signaling pathway proteins [17,18]. Therefore, SelW may be involved in regulating the association between 14-3-3 and many other target proteins. The general mechanism of regulation of this association remains to be elucidated. 14-3-3 recognizes the RSXSXP phosphor-serine motif and the RXXXSXP phosphor-threonine motif of target proteins [41,47]. TAZ contains four HXRXXS phosphorylation motifs [27,30,33]. However, SelW does not contain the phosphorylation motifs found in its well-known partners. 14-3-3 binds not only to the phosphorylation motif but also to the non-phosphorylation motif of binding partners [17,30,47,48]. SelW may interact with 14-3-3 in a redox manner [5,15,16]. The cysteine in 14-3-3 (Cys191 and Cys195 in 14-3-3 β and γ) has been identified as a SelW binding site, and residues in SelW (aa 8–22, 38–51 and 58–64) were predicted to be 14-3-3 binding regions in an NMR study [16]. Further study with a mutant SelW that disrupts the interaction with 14-3-3 remains.

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